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Original Research Article

Baicalein and U0126 suppress bladder cancer proliferation via MAPK signaling pathway

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Abstract

Purpose: *To investigate baicalein and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126)effects on human bladder cell line T24 proliferation and related mechanisms.*
Methods: *Twenty micromoles of baicalein or 10 μM U0126 were incubated with T24 cells. Cell viability was tested by CCK8 assay. Cell cycle was evaluated by flow cytometry while cell apoptosis was detected by Annexin V/PI and TUNEL assay. MAPK signaling pathway was evaluated by real time polymerase chain reaction (RT-PCR) and western blot.*
Results: *Baicalein and U0126 suppressed bladder cancer cell T24 proliferation by blocking cell cycle in G0~G1 phase. TUNEL and Annexin V/PI detection showed both baicalein and U0126 induced T24 cell apoptosis. Baicalein and U0126 significantly down-regulated MAPK signaling pathway related molecule activity in both mRNA and protein levels ($p < 0.05$).*
Conclusion: *Baicalein and U0126 restrain bladder cancer cell proliferation and promote cell apoptosis by affecting MAPK signaling pathway. Thus, they have potentials for use in the treatment of bladder cancer.*

Keywords: *Bladder cancer, Baicalein, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene, MAPK signal pathway, Apoptosis*

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INTRODUCTION

Bladder cancer is a common malignant cancer in the urinary system. Bladder epithelial tumor accounts for 95~98 %, of which transitional cell carcinoma accounts for 90 %, and non-epithelial tumor including phosphorus epithelial carcinoma and adenocarcinoma occupies 2 – 5 % [1,2]. Bladder cancer occurrence and development are multi-factorial, polygenic, and multi-step processes, which is closely related to cell proliferation and apoptosis. A variety of growth factors, oncoproteins, and their interactions play important roles in cancer proliferation and apoptosis. Their imbalance is the basis of bladder cancer oncogenesis [3,4]. Inducing cell apoptosis is an important strategy for bladder cancer treatment [5].

Mitogen-activated protein kinase (MAPK), including ERK1/2, JNK and P38, is closely related to cell proliferation, differentiation, and carcinogenesis [6]. Extracellular signal-regulated kinase (ERK) is one of the most important classical kinase signaling pathways of MAPK family. It can be activated by phosphorylation, and participates in cell proliferation, differentiation, and apoptosis by regulating a variety of oncogenes. ERK1/2 level has been

found significantly overexpressed in breast, colon and lung cancers. JNK signaling pathway could be triggered by multiple factors, including cytokines, heat shock, growth factors, osmotic pressure, stress, ionizing radiation, and oxidative damage. Activated JNK can phosphorylate c-Jun, ATF-2, and Elk, which regulate cancer cell proliferation, differentiation, and apoptosis. P38 can regulate multiple biological processes, such as inflammation, apoptosis, and cell cycle. At present, it is found that P38 has four types of isomers, including P38α, P38β, P38γ, and P38δ. Different subtypes distributes in different tissues. After activation by MKK3 or MKK6, P38 can phosphorylate ATF and Elk-1 to regulate cell proliferation, carcinogenesis, or apoptosis [7].

Because of its high incidence and fatality, anti-bladder cancer drugs investigation is full of challenge. Currently, the commonly used chemotherapy drugs in the clinic include methotrexate, doxorubicin, cisplatin, and fluorouracil. They have certain curative effect but the side effect is high. Therefore, new drug research is of great clinical significance [8]. Baicalein is a type of Chinese Traditional Medicine that is often used in the treatment of cerebrovascular disease after paralysis. It also can be adopted in cancer treatment, whereas its curative effect in bladder cancer is still poorly understood [9]. U0126 is a highly specific MEK1/2 inhibitor, which can reduce MAPK phosphorylation to inhibit cancer cell proliferation [10]. This study mainly explored baicalein and U0126 combined therapy to affect human bladder cancer T24 cell proliferation and apoptosis.

EXPERIMENTAL

Materials

Human bladder cancer T24 cells were obtained from Department Of Urinary Surgery, Wuhan Tongji Hospital. The cells were maintained in McCoy's 5A media containing 10 % fetal calf serum, 100 g/mL streptomycin, and 100 U/mL penicillin, and cultured at 37 ^{o}C and 5 % CO_2. The cells were digested by 0.25 % trypsin for passage. Baicalein purchased from Biopurify. U0126 was bought from Haoran Biotech Co, Ltd.

CCK8 assay

T24 cells in suspension were seeded in a 96-well plate. Different concentrations of baicalein or U0126 were added to the cells for 4 h in triplicate. Thereafter, 10 μL CCK8 reagent was inserted to the well and incubated for 4 h to form formazan. The samples were measured at 450 - 490 nm on a microplate reader (Thermo Electron).

Cell cycle detection

T24 cells were seeded in 12-well plate and stimulated by baicalein or U0126. After collection and washing twice with PBS, the cells were moved to 1 mL 70 % precooled ethanol at 4 ^{o}C overnight. Then the cells were washed with PBS and interfered by 100 mg/L RNase at 37 ^{o}C for 30 min. After incubated with 50 mg/L PI at 4 ^{o}C avoid of light for 30 min, the cells were detected on flow cytometry with excitation wavelength at 488 nm. The primary result was analyzed with a cell cycle matching software to record hypodiploid peak. All the experiments were repeated for at least three times.

TUNEL assay

T24 cells were washed with PBS twice after treatment and then fixed in 4 % PFA at room temperature for 10 min. Then the cells were treated with 3 % hydrogen peroxide for 10 min to inhibit endogenous catalase. After incubation with protease at room temperature for 30 min, the cells were washed with PBS twice. Next, the cells were incubated with 50 μL TUNEL at room temperature for 60 min. and observed under microscope after washing with PBS three times to analyze the result.

Annexin V/PI assay

After treating with 20 μM baicalein or 10 μM U0126 for 24 h, T24 cells were collected and washed with PBS twice. Then the cells were resuspended in 400 μL 1×binding buffer and treated by 5 μL Annexin V-FITC in the dark for 15 min. Then the cells were added with 10 μL PI and incubated avoid of light for 5 min. The cells were then tested for early and late apoptosis with a flow cytometry. The results were analyzed with Cell Quest software. Each group was repeated three times.

Real time PCR

Total RNA was extracted using Trizol and reverse transcribed to cDNA using K1622 kit (ThermoFermentas). The primers used were designed by Primer 6.0. Real time PCR was applied to test target gene expression. Reaction condition: 55 ℃ for 1 min, followed by 40 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 45 s. GAPDH was selected as the housekeeping gene. $2^{-\Delta Ct}$ was applied to calculate relative expression level. The primers sequences were listed in Table 1.

Table 1: Primer sequences

Gene name	Primer	5'-3'
ERK1/2	Sense	AATCACACGGTAGACACTGAAATGCC
	Anti-sense	CATCATCCCATCTAAAATGTCCCCTG
Bax	Sense	CCCGAGAGGTCTTTTTCCGAG
	Anti-sense	CCAGCCCATGATGGTTCTGAT
Bcl-2	Sense	GGTGGGGTCATGTGTGTGG
	Anti-sense	CGGTTCAGGTACTCAGTCATCC
P38	Sense	CCCGAGCGTTACCAGAACC
	Anti-sense	TCGCATGAATGATGGACTGAAAT
GAPDH	Sense	GCACCGTCAAGGC
	Anti-sense	TGGTGAAGACGCCAGTGGA

Western blot

T24 cells were placed on ice for 20 min after which protease inhibitor was added and lysed to extract protein. After centrifugation at 12,000 r/min, the supernatant was taken into to a new eppendorf tube and the protein concentration was determined with BCA reagent. A total of 20 μg protein was separated by 10 % SDS-PAGE and transferred to PVDF membrane. After blocking with 5 % skimmed milk for 1.5 h, the membrane was incubated with primary antibody for 2 h. Then the membrane was incubated with secondary antibody for 30 min and washed with PBST. The membrane was developed with DAB and scanned using ECL. All experiments were repeated three times.

Statistical analysis

All the data are depicted as mean ± standard deviation (SD) and were analyzed with SPSS19.0 software. Data comparison was performed using one-way ANOVA or t-test. $P < 0.05$ was considered as statistically different.

RESULTS

Baicalein and U0126 suppressed T24 proliferation by blocking cell cycle

To explore the restraining effect of baicalein and U0126 on T24 cells, we used different concentrations of baicalein (1, 2, 5, 10, 20, 50, 100, 200 and 300 μM) or U0126 (1, 2, 5, 10, 20 and 30 μM) to treat T24 cells for 24 h. CCK8 results demonstrated that T24 cell viability was significantly reduced by baicalein or U0126 with dose dependence ($p < 0.05$) (Figure 1A). Since MAPK signaling pathway can regulate cell cycle, we aimed to clarify if baicalein and U0126 impact on T24 cell cycle. We treated T24 cells with 20 μM baicalein or 20 μM U0126 for 24 h. Flow cytometry showed that compared with control, cell content increased in G_0~G_1 phase and reduced in S phase (Figure 1B). U0126 addition markedly down-regulated cell content in S phase compared with the single baicalein group (Figure 1B). We also tested for cyclin D1 mRNA expression in T24 cells treated with baicalein or U0126. Real time-PCR illustrated that cyclin D1 mRNA level declined after treatment with 20 μM baicalein for 24 h. U0126 further down-regulated cyclin D1 expression ($p < 0.05$, Figure 1C).

Baicalein and U0126 induced T24 cell apoptosis

To explore if baicalein and U0126 impact on T24 cell apoptosis, we treated T24 cells with 20 μM baicaleina and 20 μM U0126. TUNEL assay showed that both baicalein and U0126 caused T24 cell apoptosis synergistically (Figure 2A). To further examine the specific phase of apoptosis caused by baicalein and U0126, annexin V/PI staining showed that baicalein and U0126 increased both early and late phases of apoptosis with synergistic effect (Figure 2B).

Baicalein and U0126 affected MAPK signaling pathway and Bax/Bcl-2 ratio at mRNA level

Since previous research reported that baicalein can regulate MAPK signaling pathway, we tested the effect of baicalein and U0126 on MAPK signaling pathway in T24 cells at the mRNA level. Real time PCR results showed that baicalein reduced ERK1/2 and P38 levels in T24 cells. Moreover, it significantly upregulated pro-apoptotic factor Bax mRNA expression and downregulated apoptosis suppression gene Bcl-2 mRNA level, resulting in Bax/Bcl-2 ratio elevation ($p < 0.05$, Figure 3).

Baicalein and U0126 reduced MAPK signaling pathway and increased Bax/Bcl-2 ratio at protein level

Furthermore, the impact of baicalein and U0126 on MAPK signaling pathway and apoptosis related protein expression. Western blot revealed that ERK1/2 phosphorylation and P38 levels were markedly reduced in T24 cells treated with baicalein. Bax and Bcl-2 protein also showed

similar trend with mRNA expression. Baicalein together with U0126 suppressed MAPK signaling pathway related proteins expression more compared with single baicalein treatment (Figure 4).

DISCUSSION

Bladder cancer is one of the main causes of morbidity and mortality in urinary system, with 7 % morbidity and 3 % mortality among cancers [11,12]. Although localized bladder carcinomas can be treated by surgical resection, however, its recurrence and progression rates are still high. More in-depth research is needed for bladder cancer to clarify the underlying molecular mechanisms of its tumorigenesis. There is extensive information on bladder cancer at the genetic and molecular levels, however, their limited value compelled researchers to search for new molecular parameters for bladder cancer treatment.

Baicalein is the active component of Chinese traditional medicine radix scutellariae with multiple effects, such as antibiotic, antivirus, anti-inflammation. It has a variety of effects and can decrease inflammation, has antibacterial, antiviral, liver protection, diuresis, and anti-cancer properties. At present, it is mainly used for acute or chronic inflammation, while its curative effect in bladder cancer is still poorly understood. Some researchers reported that baicalein can inhibit cancer formation and induce cancer cells apoptosis, which is attractive with good prospect [13]. U0126 is an efficient selective inhibitor of MAPK kinase MEK1 and MEK2. It presents weak or no inhibitory effect on other kinases, such as PKC, Raf, and Cdk4. U0126 can suppress activated or inactivated MEK1/2 simultaneously, while PD98059 can only inhibit inactivated MEK1/2. U0126 suppresses MEK1/2 by non-competitive inhibition, as it not only antagonizes the transcriptional activity of AP-1, but also prevents the activation of MAPK p42/p44 encoded by erk2 and erk1 gene. Since MAPKp42/p44 participates in signaling cascade activated by LPS or other lipid through TLRs, U0126 could be used for numerous cancer therapy [14].

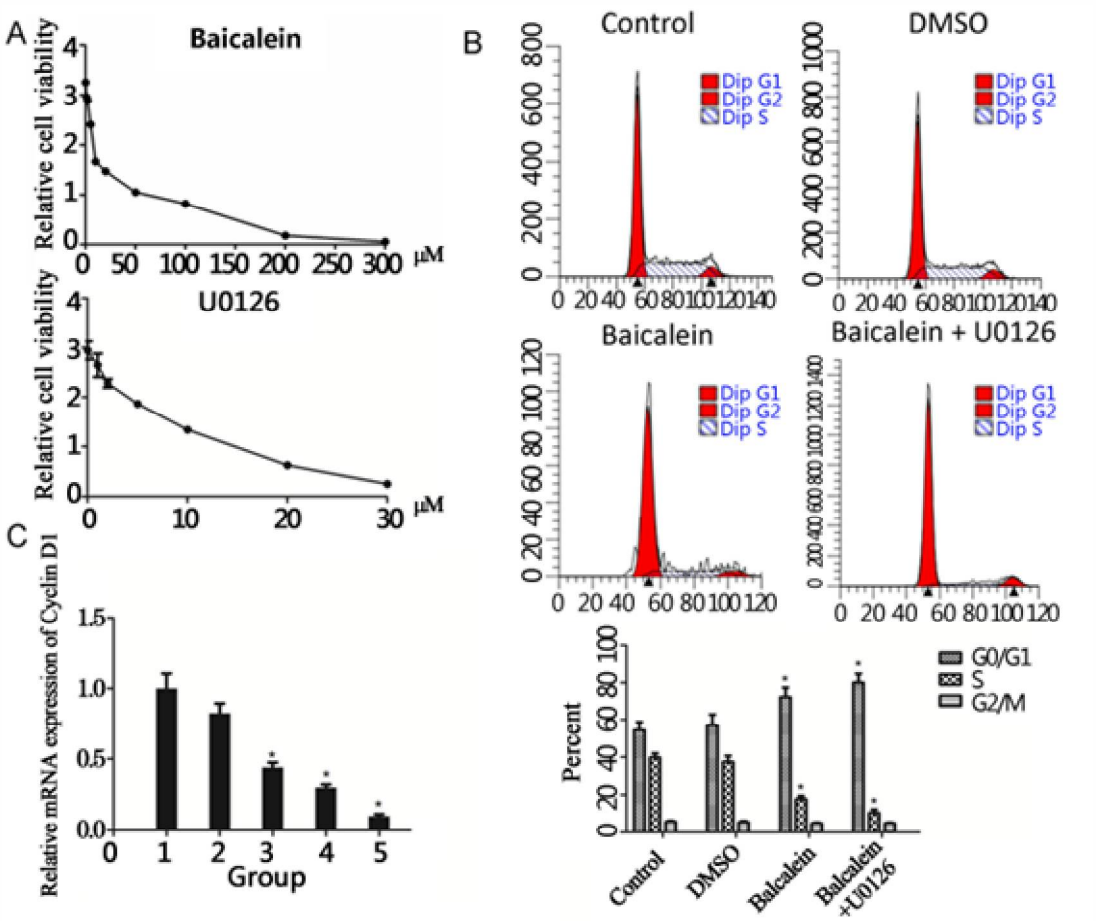

Figure 1: Baicalein and U0126 suppressed T24 cell proliferation by restraining cell cycle. (A) T24 cell viability detected by CCK-8. (B) T24 cell cycle tested by flow cytometry. (C) cyclin D1 mRNA expression determined by real time PCR. ***Note:*** 1 = control; 2 = control + DMSO; 3 = baicalein 20 μM; 4 = U0126 20 μM; 5 = baicalein 20 Mm + U0126 20 μM; * $p < 0.05$, compared with control

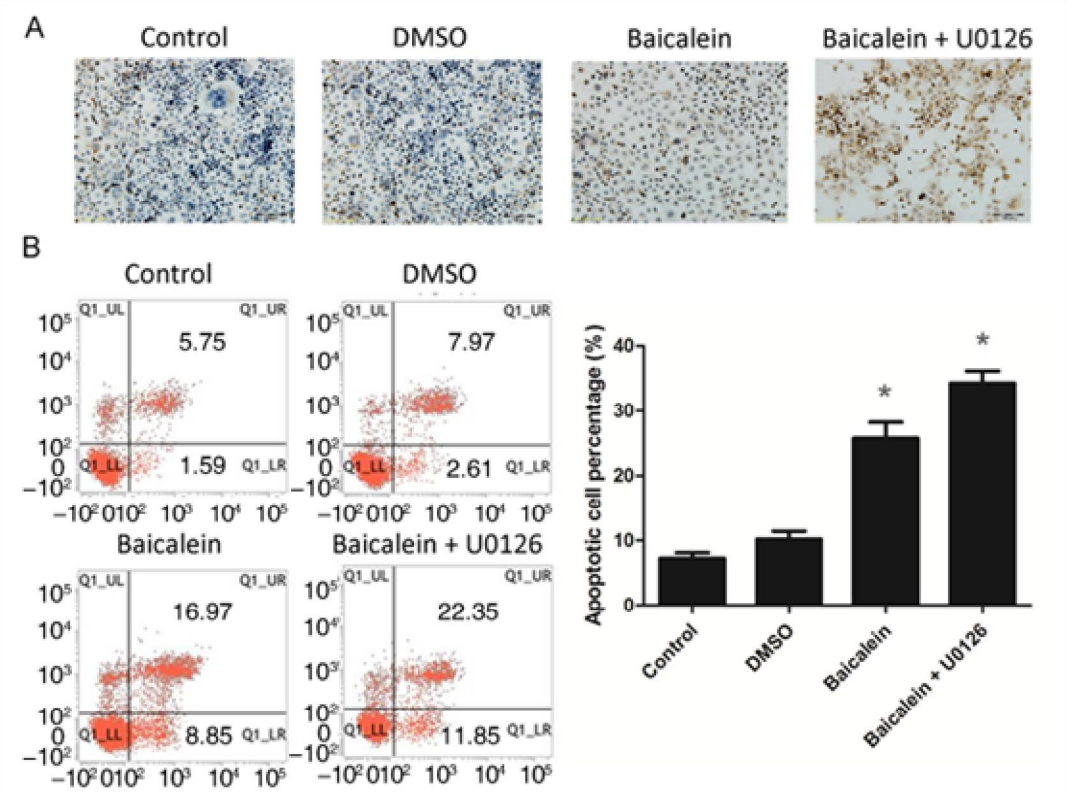

Figure 2: Baicalein induced T24 cell apoptosis. (A) T24 cell apoptosis detected by TUNEL. (B), T24 cell apoptosis tested by Annexin V/PI double staining; *$p < 0.05$, compared with control

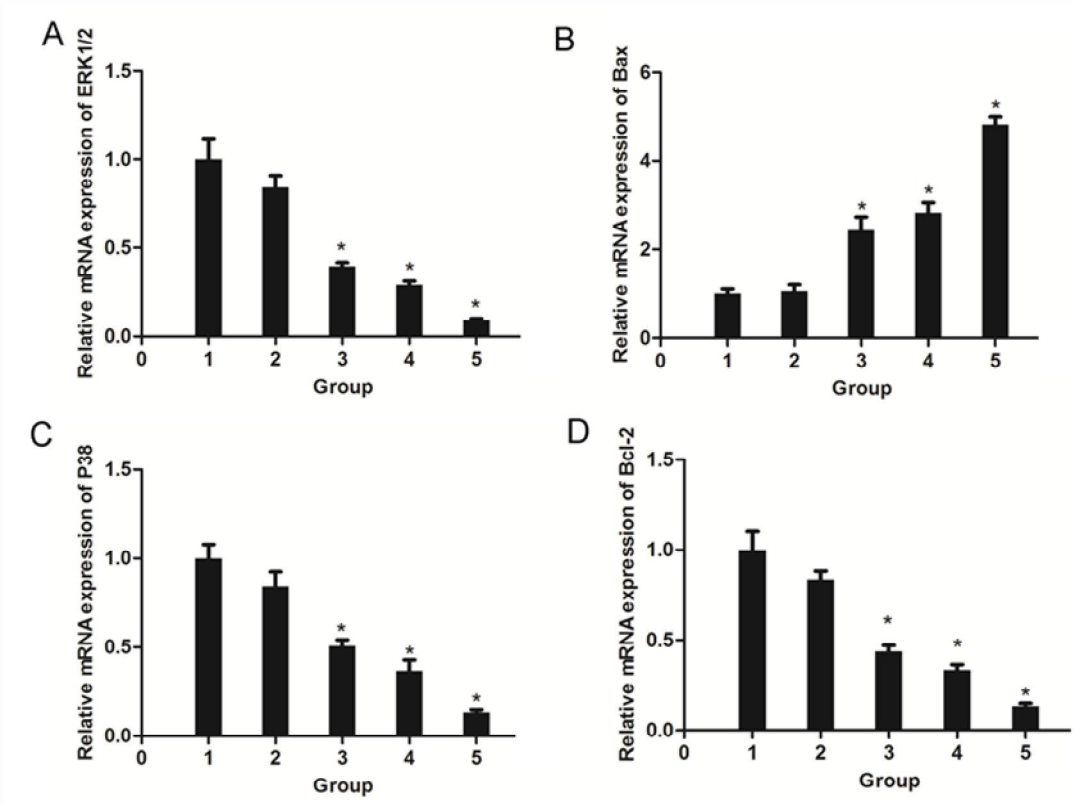

Figure 3: Baicalein and U0126 affected MAPK signaling pathway and Bax/Bcl-2 ratio at the mRNA level. ***Note:*** 1 = control; 2 = control + DMSO; 3 = baicalein 20 μM; 4 = U0126 20 μM; 5 = baicalein 20 Mm + U0126 20 μM; (A) Relative mRNA expression of ERK1/2; (B) Relative mRNA expression of Bax; (C) Relative mRNA expression of p38; (D) Relative mRNA expression of Bcl-2; *$p < 0.05$, compared with control

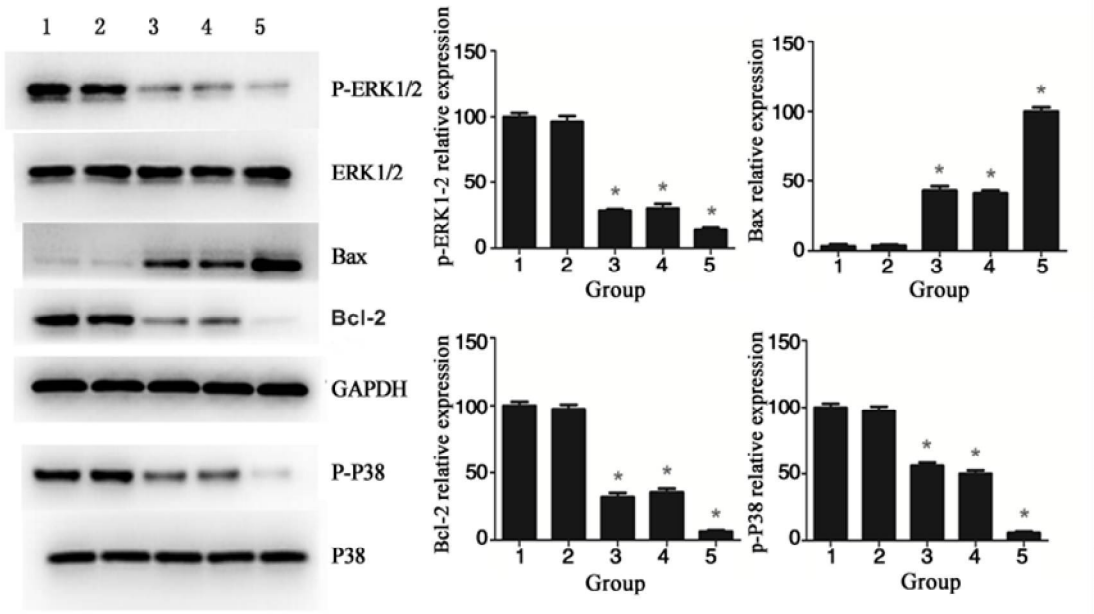

Figure 4: Baicalein and U0126 reduced MAPK signaling pathway and increased Bax/Bcl-2 ratio at the protein level. ***Note:*** 1 = control; 2 = control + DMSO; 3 = baicalein 20 μM; 4 = U0126 20 μM; 5 = baicalein 20 Mm + U0126 20 μM; *$p < 0.05$, compared with control

ERK1/2/MAPK pathway plays a critical role in bladder cancer occurrence and development. Its abnormal activation leads cancer cells to lose apoptosis and differentiation abilities, thus promoting cancer cells malignant transformation and abnormal proliferation [15]. Therefore, blocking ERK1/2/MAPK signaling pathway is of great importance for the treatment of bladder cancer. ERK1/2/MAPK signal pathway has three key molecules, namely Ras, Raf, and MEK. Ras is currently the most conservative cancer gene, including K-Ras, H-Ras, N-Ras, R-Ras, and TC21.Raf is a serine/threonine protein kinase, which contains three main types in humans as C-Raf, A-Raf, and B-Raf. MEK (ERK kinase) is an uncommon dual specific protein kinase. It is not only Tyr protein kinase, but also Ser/Thr protein kinase, including MEK1 and MEK2. The kinases that participate in ERK signaling pathway at different levels include C-Raf and MEK1/2 [16]. Inactivating these molecules as therapeutic targets may achieve the goal of bladder cancer treatment. This study applied baicalein and U0126 to inhibit human bladder cancer cell line T24, and found that ERK1/2 expression and phosphorylation level significantly decreased. It suggested that blocking ERK1/2 signaling pathway can effectively inhibit cancer cell proliferation and induce apoptosis.

ERK signaling pathway is important for regulating cell cycle by promoting cells from G1 phase to S phase [17]. Our results showed that baicalein blocked T24 cells in G0~G1 phase. Moreover, U0126 also reduced cell proportion in S phase. Cyclin D1 level was also reduced after baicalein and U0126 treatment, suggesting that both baicalein and U0126 can inhibit bladder cancer proliferation through blocking cell cycle.

P38 is another type of MAPK signaling pathway, which is closely associated with cell proliferation, differentiation, apoptosis, and carcinogenesis processes [18]. Glandular cystitis is a kind of disease characterized by hyperplasia combined with metaplasia, while the occurrence of transitional cell carcinoma is also the result of abnormal cell proliferation. Since P38 expression and phosphorylation levels declined in patients with bladder cancer, p38 signaling pathways plays a key role in the occurrence and development of bladder cancer [19]. P38 expression and phosphorylation levels was found significantly decreased in T24 cells stimulated by baicalein and U0126.

Bax and Bcl-2 are the two most important members of Bcl-2 family, which mainly distribute in the mitochondria, endothelium, and nuclear membrane. Bcl-2 can inhibit a cell under goes apoptosis through inducing resistance to various stimuli. Bax can antagonize Bcl-2 function by forming heterodimer. The ratio of Bax and Bcl-2 determines whether cell apoptosis or not [20,21]. Bcl-2 does not express in normal bladder tissue, whereas its expression rate reached 60 % in bladder cancer and increased following pathological upgrading [22,23]. Our results showed that both baicalein and U0126 induced cell apoptosis. Furthermore, they decreased Bcl-2 expression and enhanced Bax level. All of

these results suggested that baicalein and U0126 can cause bladder cancer cell apoptosis.

CONCLUSION

Taken together, baicalein and U0126 can inhibit T24 cell proliferation through regulating MAPK signaling pathway. Their combination increased the inhibition of bladder cancer cell proliferation and facilitated cell apoptosis, providing theoretical basis for their use in bladder cancer treatment.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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